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Cloning, characterization, and expression of growth regulator *CYCLIN D3-2* in leafy spurge (*Euphorbia esula*)

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We have isolated both a genomic and near full length cDNA clone for a D-class cyclin gene from the perennial weed leafy spurge. Sequence analysis indicates that this gene has the highest similarity to *CYCLIN D3-2* of Arabidopsis. This gene is preferentially expressed in growing shoot apices and is up-regulated in adventitious buds on resumption of growth following loss of correlative inhibition (apical dominance). *CYCLIN D3-2* is also induced in nongrowing adventitious buds of plants treated with gibberellic acid or after removal of leaves—treatments known to up-regulate expression of G1 to S phase transition–specific genes such as *HISTONE H3* in adventitious buds. *CYCLIN D3-2* was not induced on removal of the apical and axillary buds. Expression of *CYCLIN D3-2* is down-regulated in adventitious crown buds during initiation of ecodormancy in early winter. Sequence comparisons of *CYCLIN D3-2* with its putative orthologue from Arabidopsis identified several conserved motifs in the promoter region and a conserved region capable of forming a stable hairpin loop in the 5′ untranslated region. Conservation of these noncoding sequences across species strongly suggests they have a regulatory function.

Nomenclature: Leafy spurge, *Euphorbia esula* L. EPHES; arabidopsis *Arabidopsis thaliana* L. ARATH.

Key words: Bud dormancy, cell cycle, growth, cyclins.

Regulation of growth from adventitious buds is a critical process for controlling vegetative reproduction in perennial weeds. We are using leafy spurge as a model perennial weed to study signals regulating growth of adventitious buds. Leafy spurge is an ideal weed for studying growth of adventitious buds. It is easily cultivated in the greenhouse and small garden plots and produces large numbers of adventitious buds on its roots and subterranean stem (often referred to as the crown). Once formed, these buds enter a paradormant state that persists until they are induced to grow by loss of or separation from the aerial portion of the plant. Paradormancy is also known as apical dominance or correlative inhibition (Cline 1991). For a short period of time in the fall, both crown and root buds enter a state of endodormancy in which they loose their ability to grow, even after the aerial portion of the plant is excised or killed and the plants are moved into the greenhouse under growth-sustaining conditions. This endodormant state is released by cold temperatures (Harvey and Nowierski 1988). Adventitious buds of leafy spurge can also enter an ecodormant state that is maintained during winter by extreme cold and drought. Thus, underground adventitious buds display all three types of dormancy, endo-, eco-, and paradormancy as described by Lang et al. (1987). An expressed sequence tag database containing $>$ 30,000 sequences has been established from a normalized, whole-plant cDNA library. Additionally, both a two-hybrid cDNA library from growing buds and a genomic expression library are available for this species.

Paradormancy of the adventitious buds located on the roots and crown of leafy spurge plants (often referred to as root buds or crown buds) has been well characterized and shown to be influenced by two separate signals. One growth-inhibiting signal is produced in the mature photosynthesizing leaves and its production and transport is inhibited by darkness or lack of carbon fixation (Horvath 1999). This leaf-derived signal acts through gibberellic acid (GA) to inhibit the G1 to S phase transition of the cell cycle in root buds of leafy spurge (Horvath et al. 2002). The other signal requires active polar auxin transport, and loss of this signal is required for completion of the cell cycle and continued growth and development but does not appear to influence the G1 to S phase transition (Horvath et al. 2002). The study of endodormancy in crown buds of leafy spurge has only recently been initiated, although there is speculation that chromatin remodeling mechanisms might play a role in this phenomenon (Horvath et al. 2003). The mechanisms influencing ecodormancy in leafy spurge have not yet been addressed, although the actions of cyclin-dependent kinase (CDK) inhibitors, such as *ICK1*, are likely to play a role (Horvath et al. 2003).

In plants (and animals), one of the primary mechanisms of growth regulation is through control of cell division. Initiation of cell division in nongrowing or dormant cells occurs when cells are stimulated by growth hormones. In plants, the initiation of G1 is marked by the induction of a small family of G1 cyclin genes designated as D-class cyclins (Mironov et al. 1999). Cyclin D genes are induced by several plant hormones such as cytokinin, brassinosteroids, and GA, all of which have been associated with growth induction (Ogawa et al. 2003; Riou-Khamlichi et al. 1999). In at least one system, cytokinin is able to induce cyclin D expression without the need for de novo protein synthesis (Hu et al. 2000). This observation suggests that cyclin D induction might be directly responsive to the signal transduction mechanisms regulating growth and is likely to be among the first genes induced on perception of growth-inducing signals such as cytokinin.

Cell division results in the production of two daughter cells unless additional signals prevent completion of the cell cycle. In both plants and animals, cell cycle progression can be blocked at either the G1 to S phase transition or at the G2 to M phase transition (den Boer and Murray 2000). Progression through the G1 to S phase transition requires both the expression of cyclin D and a CDK. Together, these proteins form a complex that phosphorylates the retinoblastoma protein (Rb). Once Rb is hyperphosphorylated, it releases several transcription factors and can itself be involved in chromatin remodeling events that are required for initiation of DNA replication (Dewitte and Murray 2003; Shen 2002).

To identify early signals regulating the reinitiation of growth in adventitious buds of leafy spurge, we have focused on cloning and identifying the regulatory mechanisms controlling cyclin D expression. In this paper, we describe the initial cloning and expression analysis of *CYCLIN D3-2* from leafy spurge. We also present sequence analysis comparisons that identify potential regulatory elements that are conserved between the *CYCLIN D3-2* genes of arabidopsis and leafy spurge.

Materials and Methods

Plant Material and Treatments

Plants for all experiments were grown in the greenhouse as single stems in cones (5 by 20 cm) with Sunshine mix[1] under a 16-h day with mixed natural and artificial lighting or in garden plots (for seasonal studies). All experiments presented were independently repeated on at least two sets of plants.

To study the temporal expression pattern of *CYCLIN D3-2* during loss of paradormancy and disturbance, the plants were removed from their growth containers and either defoliated by excising the aerial portion of the plant or left intact and placed back into their containers. Adventitious buds from the crown of the plants were collected at 0, 24, 48, and 72 h after treatment. To avoid potential artifacts caused by circadian effects, all buds were harvested at the same time of day.

To test the effects of cold stress on gene expression, plants were placed in a growth chamber at a constant temperature of 5 C under a 16-h photoperiod. Mature leaves and shoot apices were harvested separately from 21 individual plants 5 to 6 d after initiation of cold stress.

To study the effects of various plant organs on root bud growth, specific plant organs were removed as previously described (Horvath 1999). Briefly, plants either were left intact or had the entire aerial portion of the plant excised to the base of the crown. Alternatively, the top 10 cm of the plants were removed (meristemless) and were stripped of either mature leaves (leafless) or axillary buds (budless). All distinguishable underground buds $\geq$ 0.25 mm were harvested 2 d after treatment.

To test the effects of GA on gene expression in root buds, plants were watered once with 25 ml of a 0.5% TWEEN 20 solution[2] with or without 1 mM GA_3 (potassium salt).[3] All distinguishable underground buds $\geq$ 0.25 mm were harvested 3 d after treatment.

To follow seasonal effects on gene expression, plants grown outdoors were examined at specified times, and all crown buds were harvested. This process was repeated over a 4-yr period starting from 2000 through 2004.

Cloning and Characterization of *CYCLIN D3-2*

Total RNA was isolated from growing root buds of leafy spurge. This RNA was used in a reverse transcription polymerase chain reaction (RT-PCR) with primers[4] designed to amplify a conserved region of cyclin D genes. Primer sequences were GCTGTTGATTGGATGCTTARRGT (5′) and TGAGTTTCTTCAACTTTAGCAGC (3′), and annealing temperature was 45 C; the amplification was carried out over 30 cycles. This reaction resulted in the amplification of several bands, but only one (faint band) was the predicted 182-bp length. This band was gel purified and then reamplified with the same primers and under the same conditions. Sequence data confirmed the DNA band was from a cyclin D3 gene, and this fragment was used as a probe to identify the cDNA gene from a lambda hybrid-Zap2 cDNA library made from adventitious buds of leafy spurge that were collected 3 d after defoliation. An amplified 5′ fragment of the cDNA was used as a probe to identify the genomic clone from a lambda Zap-Express genomic library made from leafy spurge. Gene identity was confirmed by sequencing rescued plasmids that hybridized to the respective probes.

Sequence data from the genomic and cDNA clones were compared with similar regions from the Arabidopsis *CYCLIN D3-2* gene with Web-based programs designed to identify putative conserved *cis*-acting elements and homologies to known plant *cis*-acting sequences (Higo et al. 1999; Lescot et al. 2002). Potential secondary structure of the 5′ untranslated region (UTR) was predicted by the program available at (The GeneBee Group 2001).

Nucleic Acid Hybridization

RNA was collected by the pine tree extraction method (Chang et al. 1993). Total RNA was separated on denaturing agarose gels and blotted according to standard techniques (Sambrook et al. 1989). DNA probes were prepared by PCR amplification of designated cDNAs followed by isolation of the resulting band after separation on agarose gels. The probe for *CYCLIN D3-2* was amplified primarily from the 3′ noncoding portion of the gene. This probe did not hybridize to a near full length *CYCLIN D3-1* gene which was isolated during the cloning of *CYCLIN D3-2*. The probe for *HISTONE H3*, which is an accepted S-phase marker of the cell cycle and served here as a positive control for cell cycle induction was previously described (Horvath et al. 2002). Radiolabeled probes for the genes were prepared and hybridized to the various blots (5× sodium chloride–sodium citrate [SSC]/50% formamide[5] at 42 C). Blots were washed four times at room temperature in 2× SSC, 0.2% sodium dodecyl sulfate for 5 min each and then two times at 65 C for 15 min each. The resulting hybridizations were visualized by autoradiography or on a Packard Instant Imager.[6] Linearity was maintained for all of the images presented.

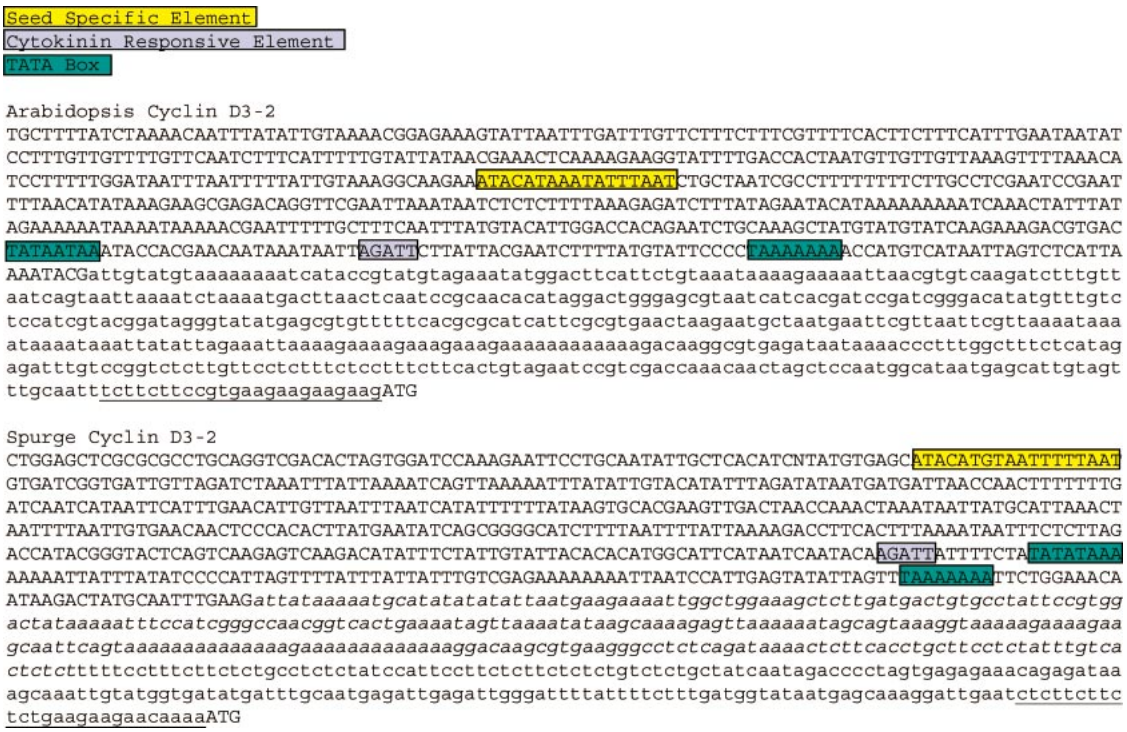

FIGURE 1. Sequence comparison between *CYCLIN D3-2* genes from Arabidopsis and leafy spurge. Conserved elements are shown in colored boxes corresponding to their putative functions as shown. Transcribed regions are depicted as lowercase letters, with sequences capable of forming stable hairpin loop underlined. Italicized lowercase letters in the sequence of the leafy spurge *CYCLIN D3-2* gene are putatively transcribed on the basis of comparisons of the genomic clone with the Arabidopsis *CYCLIN D3-2* cDNA but were not found in any cDNA clone.

Results and Discussion

Cloning and Characterization of *CYCLIN D3-2* from Leafy Spurge

Nondegenerate primers were designed from the consensus sequences of nearly 20 different cyclin D genes from multiple plant species. RT-PCR with these primers produced a band of the predicted size from RNA isolated from growing adventitious buds. Sequence analysis of the amplified fragment confirmed it was derived from a D-class cyclin. This fragment was then used as a probe to isolate a cDNA clone (GenBank AY340588) and genomic clone (GenBank AY542268) from leafy spurge libraries. Analyses of the sequence data from these clones suggested that both were most similar to a cyclin D3 gene from poplar (*Populus alba*). The cDNA clone contained the entire coding sequence plus 201 bp of the 5′ UTR sequence and was most similar to the *CYCLIN D3-2* member of the cyclin genes of Arabidopsis. Multiple attempts to obtain a full-length cDNA with 5′ rapid amplification of cDNA ends or by extensive screening of the cDNA library proved ineffective. The genomic clone was > 99% identical to the cDNA for *CYCLIN D3-2* throughout the predicted coding sequence and contained approximately 600 bp of putative promoter sequences. Because leafy spurge is an auto-allo hexaploid (Schulz-Schaeffer and Gerhardt 1987), the minor differences between the genomic and cDNA clones could be from cloning artifacts or the cloning of different copies of the same gene from two of the genomes. The complete genomic clone for *CYCLIN D3-2* contained three predicted introns and a predicted 5′ UTR of approximately 475 bp.

CYCLIN D3-2 Genes Contain Conserved Sequence Motifs in Both Their Promoters and 5′ UTR

Phylogenetic footprinting has been used to identify numerous *cis*-acting elements in the regulatory regions of various genes (Arguello-Astorga and Herrera-Estrella 1998; Horvath and Olson 1998; Tagle et al. 1988). Thus, we compared the Arabidopsis and leafy spurge genes encoding *CYCLIN D3-2* and identified several conserved motifs (Figure 1). We identified a putative *cis*-acting element described as a seed-specific transcription factor binding site located 487 bp upstream of the predicted TATA box in the leafy spurge *CYCLIN D3-2* gene. A similar sequence was located 312 bp upstream of the TATA box in the Arabidopsis *CYCLIN D3-2* gene. Additionally, there was a putative cytokinin responsive element located 105 and 41 bp upstream of the TATA boxes in the leafy spurge and Arabidopsis genes, respectively. Interestingly, both genes contained atypical TATA box motifs "TAAAAAAA" which, at least in Arabidopsis, act as the functional transcription initiation complex binding site. This sequence was preceded by a typical TATA box motif "TATA(T/A) (T/A)AA" located 90 and 71 bp upstream of the TATA boxes in the leafy spurge and Arabidopsis genes, respectively. Additional conserved sequences located in the 5′ UTR of both genes include several stretches of AG and CT sequences, which might form similar hairpin structures. In both species, one short region of AG and CT sequences is located immediately upstream of the start of translation. Interestingly, this potential hairpin sequence from the Arabidopsis *CYCLIN D3-2* gene was identified as a potential micro RNA binding site by the program written by Adai et al. (2005). Finally, both genes contained the same number

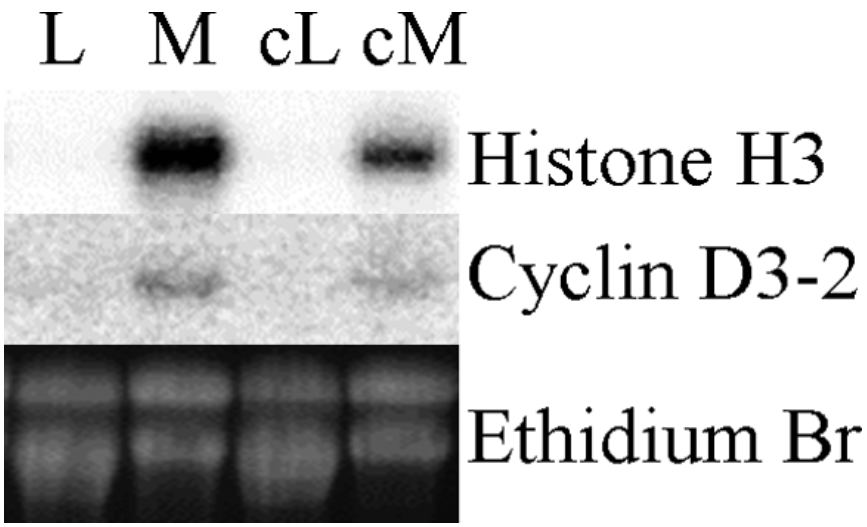

FIGURE 2. Expression of *CYCLIN D3-2* in growing shoot apices and mature leaves. RNA was collected from leaves (L) and shoot apices (M) of leafy spurge growing at normal temperature or after 5 d at 5 C (cL and cM). Blots were hybridized to the designated probes. Ethidium-stained gel is shown as a loading control.

of introns and exons located in nearly identical positions within the coding regions of the genes (data not shown).

CYCLIN D3-2 Is Preferentially Expressed in Growing Tissues

RNA isolated from growing shoot apices and leaves, as well as from cold-treated shoot apices and leaves, was subjected to northern blot analysis to determine whether *CYCLIN D3-2* was preferentially expressed in growing tissues (Figure 2). Even though the number of actively dividing cells from an individual shoot was limited, the results clearly demonstrated that the *CYCLIN D3-2* gene was expressed more abundantly in growing tissues such as the shoot apices than in nongrowing tissues such as mature leaves. Cold treatment slightly reduced the level of *CYCLIN D3-2* expression in the shoot apices. In all cases, the induction of *CYCLIN D3-2* was qualitatively similar to, but displayed less robust differential expression than, the *HISTONE H3* gene used as a positive control.

CYCLIN D3-2 Is Induced within 48 h after Growth Induction in Root Buds of Leafy Spurge

If *CYCLIN D3-2* is directly responsive to signals regulating dormancy, it could serve as a possible tool for identifying components of the signal transduction machinery involved in dormancy regulation. To determine how responsive *CYCLIN D3-2* expression was to growth induction in adventitious buds, root buds were collected at various times after growth induction by excision of the aerial portion of the plant. RNA from these buds was subjected to northern analysis (Figure 3). Results of these experiments indicated that in at least four different samplings (not all data shown), an increase in *CYCLIN D3-2* was consistently observed within 48 h after treatment and achieved maximal expression between 3 and 4 d later. In all experiments, slight increases in *HISTONE H3* expression was observed after, or concurrent with, the initial increase in *CYCLIN D3-2* expression.

Although *CYCLIN D3-2* was up-regulated after removal of the aerial portion of the plant, it did not appear to be rapidly up-regulated. In both plants and animals, D-class cyclin genes are directly responsive to perception of growth-regulating hormones and are among the first genes to be induced on release of the cells from dormancy (den Boer and Murray 2000). Indeed, in plants, cyclin D3 expression can be induced by cytokinin in the presence of cycloheximide, indicating that de novo protein synthesis is not required for expression (Hu et al. 2000). However, it should be noted that some hormones such as brassinosteroids can induce cyclin D3 expression through a protein synthesis–dependent pathway (Hu et al. 2000). Thus, it is possible that *CYCLIN D3-2* was up-regulated by signaling systems that were more complex than the cytokinin induction pathway. Alternatively, it is possible that there was a substantial lag time between growth induction and perception of cytokinin in the adventitious buds following loss of the aerial portion of the plant.

The induction of *CYCLIN D3-2* after defoliation in adventitious buds of leafy spurge was consistent with previous observations that maximal cell cycle activity (as shown by *HISTONE H3* expression) did not occur prior to 24 h after defoliation (Horvath et al. 2002). Thus, other signals must be needed to maintain the growth response. Shimizu-Sato and Mori (2001) hypothesized that loss of apical dominance initiates physiological changes within the stem. These changes eventually result in alteration of growth-regulating hormones that initiate growth induction after they are transported into the buds. The induction of *CYCLIN D3-2* in leafy spurge was surprisingly slower than the rate of cell cycle induction in axillary buds of pea, in which cell cycle activity was induced within a few hours of defoliation (Devitt and Stafstrom 1995). The difference in lag time observed between defoliation and maximal initiation of the cell cycle in adventitious buds of leafy spurge and axillary buds of pea suggests that these processes could be fundamentally different or that there are additional controls regulating adventitious bud growth that are not acting to regulate growth of axillary buds in other plants.

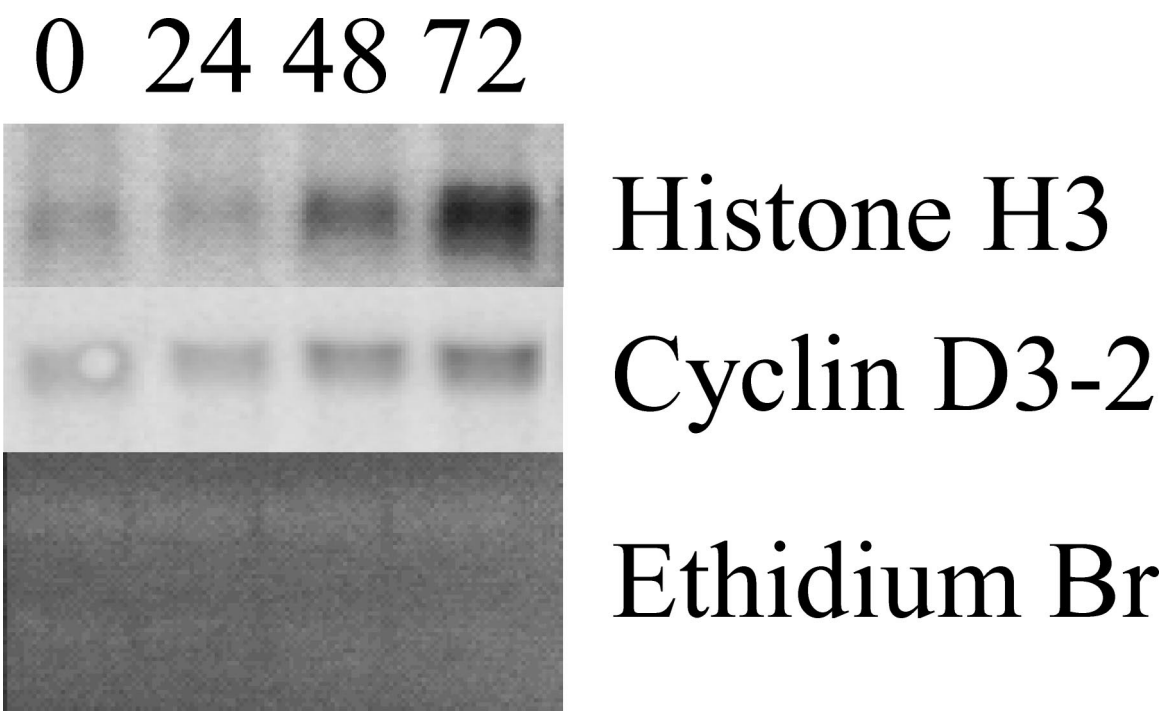

FIGURE 3. *CYCLIN D3-2* is up-regulated within 48 h after defoliation. RNA from indicated time points was subjected to northern analysis and hybridized to the designated probes. A representative ethidium bromide–stained gel is shown as a loading control.

Expression of *CYCLIN D3-2* Is Influenced by Cold Stress, GA Treatment, and Leaf Removal but Not by Loss of Growing Shoot Apices

Previous studies have shown that a signal produced in the mature leaves acts through GA to influence the G1 to S phase transition in dormant root buds of leafy spurge (Horvath et al. 2002). Also, the regulation of *CYCLIN D3* by GA has been observed in other plant systems (Ogawa et al. 2003). We have collected RNA from the root buds of intact plants that had been either treated with GA or stripped only of their mature leaves (Figure 4, Lanes G and l). Both treatments were shown to induce S phase transition in the buds, as indicated by an increase in *HISTONE H3* expression, but neither treatment was shown to be sufficient to induce sustained growth and development of the buds (Horvath et al. 2002). Additionally, we have collected RNA from root buds of plants that were stripped of all growing apical and axillary buds (Figure 4, Lane b). In previous experiments, removal of just the aerial buds did not induce sustained growth or *HISTONE H3* expression in the root buds of the plant. However, bud removal was sufficient for breaking dormancy

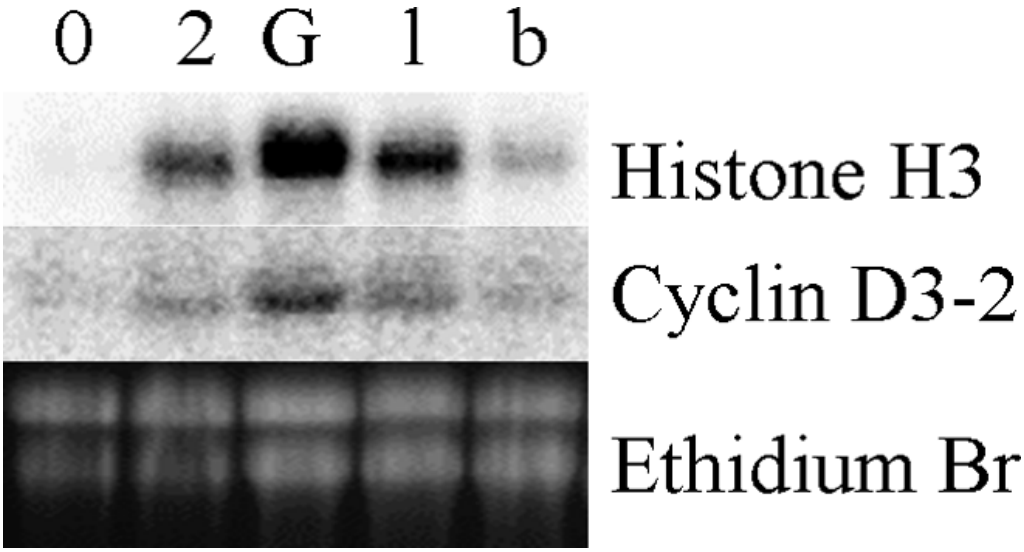

FIGURE 4. Expression of *CYCLIN D3-2* after full growth or S-phase induction as shown by expression of *HISTONE H3*. Adventitious buds were collected from intact control plants (0), from plants 2 d after growth induction by defoliation (2), from intact plants 3 d after gibberellic acid treatment (G), and from plants 2 d after excision of all leaves (l) or apical and axillary buds (b). RNA from these samples was subjected to northern analysis and hybridized to the designated probes. The ethidium bromide–stained gel is shown as a loading control.

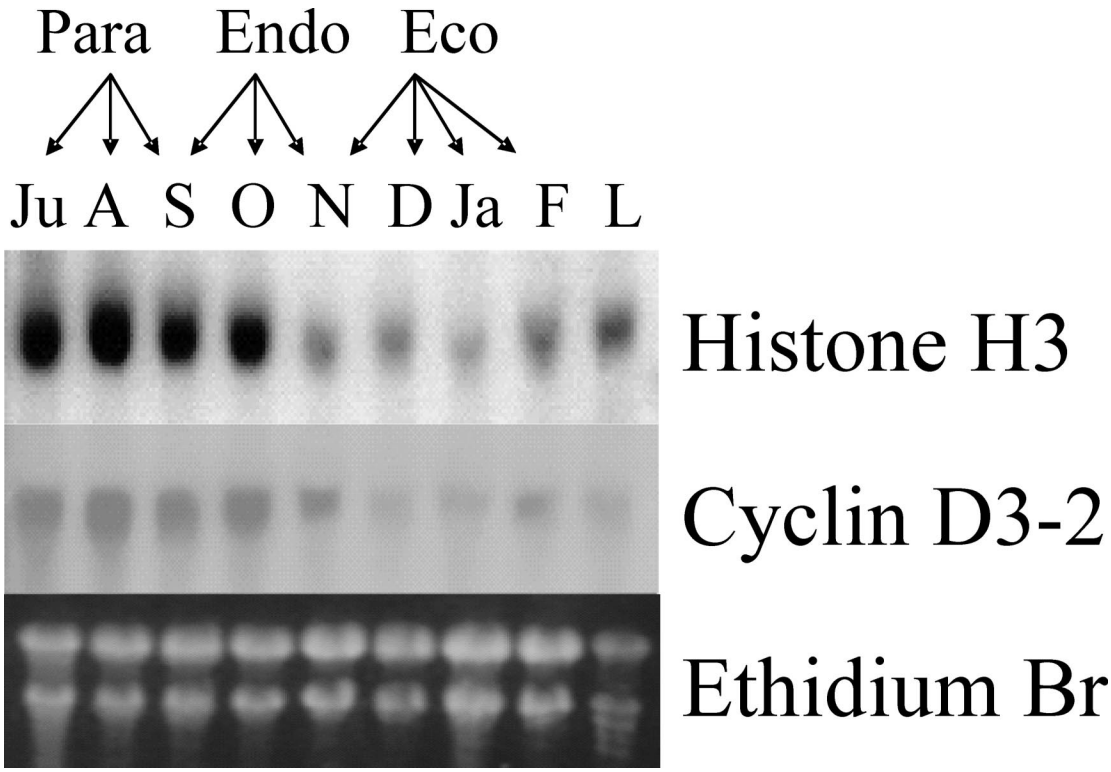

FIGURE 5. Seasonal regulation of *CYCLIN D3-2*. Crown buds of leafy spurge were collected from intact plants in July (Ju), August (A), September (S), October (O), November (N), December (D), January (Ja), and February (F). The various types of dormancy acting on the buds are shown. RNA from these samples were subjected to northern analysis and hybridized to the designated probes. The ethidium bromide–stained gel is shown as a loading control.

in plants previously treated with GA or that had been stripped of mature leaves (Horvath 1999). Nongrowing (Figure 4, Lane 0) and growing (Figure 4, Lane 2) root buds were collected as controls. Expression of *CYCLIN D3-2* was analyzed in these samples. The results clearly show that the level of *CYCLIN D3-2* mRNA was increased in GA-treated and leafless plants, but not in response to loss of auxin from the apical and axillary buds.

CYCLIN D3-2 Is Differentially Expressed in Dormant Buds during the Growing Season

Experiments (Figures 3 and 4) have shown that *CYCLIN D3-2* is regulated by conditions affecting paradormancy in root buds under greenhouse conditions. However, in the wild, root and crown buds of leafy spurge are also regulated by signals that maintain both endodormancy and ecodormancy throughout the fall and winter. To determine whether *CYCLIN D3-2* expression was controlled by physiological factors that inhibit growth during endo- or ecodormancy, crown buds of leafy spurge were collected from intact plants at various times throughout the year. Surprisingly, northern analysis of RNA from these buds showed that both *CYCLIN D3-2* and *HISTONE H3* were expressed at relatively high levels throughout the growing season when it was assumed that buds were paradormant (Figure 5). Both genes were also expressed in October, a time in which the buds are known to be in endodormancy (unpublished data). However, expression of *CYCLIN D3-2* and *HISTONE H3* was dramatically reduced when bud growth was inhibited by ecodormancy.

Northern analysis of *CYCLIN D3-2* and *HISTONE H3* expression in the growing shoot apices also demonstrated some down-regulation of these genes in response to cold temperatures (Figure 2). These results allow speculation that ecodormancy might also inhibit growth at the G1/S phase transition. Relatively high levels of expression of *CYCLIN D3-2* and *HISTONE H3* from July through October suggest that growth inhibition during the growing season and through endodormancy in October might be blocked after S phase. However, it should be noted that although *CYCLIN D3-2* is transcribed, it is possible that additional post-transcriptional regulatory mechanisms could influence its translation or activity. Indeed, the conserved hairpin loops near the start codon in the transcript strongly suggest that some additional regulatory mechanisms could be effecting cyclin expression. Additional research is needed to elucidate these intriguing observations.

Conclusion

Cyclin D3 is an important indicator of cell cycle initiation in both plants and animals (Dewitte and Murray 2003). Expression analysis of *CYCLIN D3-2* in leafy spurge indicates that it is preferentially expressed in actively growing tissues. Also, it is induced by loss of leaves and by GA treatment. Both conditions are known to initiate the G1 to S phase progression in leafy spurge buds without allowing completion of the cell cycle through the G2/M restriction point (Horvath et al. 2002). These observations strongly suggest that *CYCLIN D3-2* is functioning as a G1 cyclin in leafy spurge. Study of the regulation of *CYCLIN D3-2* should provide significant insight into the mechanisms controlling growth of buds in response to various physiological

signals regulating dormancy. Such information will lead to the identification of potential novel target sites for development of weed control strategies and compounds.

Sources of Materials

[1] Sunshine mix potting soil, Fisons Horticulture Inc., 110 110th Ave. N.E., Suite 490, Bellevue, WA 98004.

[2] TWEEN 20 (P1379), Sigma-Aldrich Co., P.O. Box 952968, St. Louis, MO 63195.

[3] GA_3 potassium salt (G1025), Sigma-Aldrich Co., P.O. Box 952968, St. Louis, MO 63195.

[4] Primers, Integrated DNA Technologies, 1710 Commercial Park, Coralville, IA 52241.

[5] Formamide (F7508), Sigma-Aldrich Co., P.O. Box 952968, St. Louis, MO 63195.

[6] Packard Instant Imager, Packard Instrument Company, 2200 Warrenville Road, Downers Grove, IL 60515.

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